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TARGETING POLO-LIKE KINASE 1 IN PEDIATRIC LEUKEMIA

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Front cover shows a leukemic cell with a monopolar spindle that undergoes apoptosis after Plk1 depletion.
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Targeting polo-like kinase 1 in pediatric leukemia

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Моїм батькам. Дякую за натхнення почати цей шлях.

To my parents. Thank you for the encouragement to start.

To my amazing nieces. Thank you for inspiration to move on.

Motivation is more important in science than anything else,
because if you are motivated, you can learn anything.

Sydney Brenner

ABSTRACT

Acute lymphoblastic leukemia (ALL) is one of the most common cancers among children in the world, with almost 90% complete remission rate after the primary treatment. However, some patients are resistant to treatment and some relapse later. Moreover, existing treatments are toxic and induce life-long adverse effects. Potentially, it can be avoided if a more specific drug is used. Therefore, there is a need for new therapeutic targets and reliable validation of the developed drugs to avoid potential off-target effects. The family of serine-threonine polo-like kinases (Plk) is important for cell cycle. Among other family members, Polo-like kinase 1 is a crucial regulator of mitosis, and it is particularly important for the proliferation of cancer cells This thesis aimed to improve understanding of Plk1 as a treatment target in pediatric leukemia.

In **Paper I** we aimed to identify the potential off-target proteins of Plk1 small-molecule inhibitors such as BI2536, volasertib and NMS-1286937. The drugs are already involved in the late-stage clinical trials and result in severe side-effects that may affect the survival of the patients. We used cellular thermal shift assay and thermal proteome profiling to identify the proteins that have a change in the thermal stability after treatment. We found that the BI2536 and volasertib bind to Prostaglandin reductase 2 (PTGR2), an enzyme that regulates prostaglandin E_2 metabolism and contributes to immune response. Also, we found that volasertib and NMS-1286937 affect the stability of a number of transcriptional coactivators, RNA splicing regulators, and proteins involved in the intracellular transport.

In **Papers II** and **III** we investigated the potential of Plk1 as a treatment target in primary cells from pediatric T-cell and B-cell ALL. We showed that Plk1 is highly expressed both in patient cells and T/B-cell ALL cell lines, compared to peripheral blood mononuclear cells (PBMCs) from healthy donors. Further we targeted primary patient cells with RNAi prodrugs – short interfering RiboNucleic Neutrals (siRNN). We showed that siRNNs entered leukemic cells and induced specific Plk1 mRNA knockdown followed by double-strand DNA breaks, cell cycle arrest and apoptosis. We also showed that siRNNs did not induce apoptosis in PBMCs compared to Plk1 small molecule inhibitor volasertib.

To conclude, small molecule inhibitors of Plk1 affect high number of proteins apart from Plk1 itself that might explain the side effects of the treatment. Specific RNAi -based drugs may help to overcome this issue in T-cell and B-cell ALL.

LIST OF SCIENTIFIC PAPERS

I. **O. Goroshchuk**, E. Kunold, L. Vidarsdottir, A. Azimi, R. Jafari, C. Palm-Apergi, *Investigating the target landscape of Plk1 small molecule inhibitors using thermal proteome profiling.* Manuscript.

II. I. Kolosenko, E. Edsbäcker, A.-C. Björklund, A.S. Hamil, **O. Goroshchuk**, D. Grandér, S.F. Dowdy, C. Palm-Apergi, *RNAi prodrugs targeting Plk1 induce specific gene silencing in primary cells from pediatric T-acute lymphoblastic leukemia patients*, J. Control. Release. 261 (2017) 199–206. doi:10.1016/j.jconrel.2017.07.002

III. **O. Goroshchuk,** L. Vidarsdottir, A.-C. Björklund, A.S. Hamil, I. Kolosenko, S.F. Dowdy, C. Palm-Apergi, *Targeting Plk1 with siRNNs in primary cells from pediatric B-cell acute lymphoblastic leukemia patients*, Sci Rep 10, 2688 (2020). doi: 10.1038/s41598-020-59653-5

LIST OF PAPERS AND MANUSCRIPTS NOT INCLUDED IN THE THESIS

I. **O. Goroshchuk**, I. Kolosenko, L. Vidarsdottir, A. Azimi, C. Palm-Apergi, *Polo-like kinases and acute leukemia*, Oncogene (2019) 38:1-16. doi:10.10387s41388-018-0443-5.

II. L. Vidarsdottir, **O. Goroshchuk,** I. Kolosenko, C. Palm-Apergi, *Designing siRNA and evaluating its effect on RNA targets using qPCR and western blot,* Methods in Molecular Biology (MIMB) volume 2036: 53-72. doi. org/10.1007/978-1-4939-9670-4_3

CONTENTS

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
BM	Bone marrow
CDK	Cyclin – dependent kinase
CETSA	Cellular Thermal Shift Assay
CpG	Cytosine phosphate Guanine
CR	Complete remission
DLT	Dose – limiting toxicity
dsRBP	Double-strand RNA binding protein
EFS	Event – free survival
FDA	U.S. Food & Drug Administration
HeH	High hyperdiploidy
MDC1	Mediator of DNA damage checkpoint 1
MTX	Methotrexate
NOPHO	Nordic Society of Pediatric Haematology and Oncology
OS	Overall survival
PARP	Poly (ADP ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PEG	Polyethylene glycol
PGE_2	Prostaglandin E_2
Plk	Polo-like kinase
PTGR2	Prostaglandin reductase 2
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNN	Ribonucleic neutral
SATE	S- acyl-2-thioethyl
TLR	Toll – like receptors
TPP	Thermal Proteome Profiling
WBC	White blood cells

1 INTRODUCTION

1.1 History of pediatric leukemia

For centuries doctors, philosophers and scientists were describing cases where people with the initial symptoms of tiredness, depressive mood, enlarged spleen and liver, progressive weakness, and bleedings were dying after several months, rarely years, from the onset of their symptoms. Despite that any person could become sick, children were considered disseminators of infections, so they usually were not admitted to the hospitals to the same extent as adults.[1]

The development of microscopy and medical science in the 18th and the 19th centuries led to publications of cases that could be potentially identified as leukemia. Finally, in 1858 Rudolph Virchow and John Hughes Bennet came to conclusions for which they became accredited for defining "leukemia" as a separate clinical entity. They succeeded to correlate and connect the clinical symptoms to the microscopical findings in the blood of the patients. The scientific discoveries of the 19th century, important for understanding of leukemia, continued.

Ernst Neumann, a professor of Pathological Anatomy at Königsberg in 1860-80s, showed that erythropoiesis and lymphopoiesis take place in bone marrow (BM). [1] In 1877, while still being a medical student, Paul Erlich established the use of triacid stain on a thin dried film formed from a blood drop. By such staining Erlich was able to distinguish between different types of cells and cellular components in the blood, and later he introduced a new classification of leukemia, dividing it into lymphoid (non-granular cells) and myeloid (granular cells) groups. Moreover, Erlich was the first to describe and implement a concept of a stem cell as a hematopoietic ancestor for other blood cells. In 1900 Naegeli, a Swiss hematologist, defined a lymphoblast as a precursor to lymphocytes. Altogether, these findings made the basis for the classical diagnostic criteria of acute leukemia: circulating lympho- or myeloblasts.

Further, the development of epidemiology has facilitated the possibility to improve the knowledge about incidence of leukemia, particularly in children. In 1904, Frank Churchill, a physician from Chicago collected history about 15 cases that were regarded as "acute lymphatic leukemia" [2] with a detailed physical examination of the patients, their blood analysis and autopsy results. He showed that the disease affected newborns and older children, pointed out that symptoms manifested during the period of several days to several months, and also described the treatment options available at that time. Finally, Gordon Ward collected records of 1457 cases of different leukemia patients and in 1917 concluded that the acute form affects mostly children with a peak incidence between 0 and 5 years of age. [1,3]

As it became known that leukemia originates in the BM, doctors worked on a development of the instruments and techniques to perform sternal or tibial BM aspiration. [4] For example, in 1929 Arinkin, a Russian doctor, reported about 103 successful sternal punctures, and this procedure was accepted to diagnose hematological malignancies. [4,5] Nowadays, investigation of the BM biopsy with further immunophenotyping, molecular and cytogenetic profiling is crucial for clinical diagnostics of leukemia in order to choose the suitable treatment strategy. [6]

1.2 Acute lymphoblastic leukemia

In 2016, The World Health Organization introduced an updated classification of tumors of hematopoietic and lymphoid tissues, [7] where acute lymphoblastic leukemia (ALL) is divided into two main groups: B-cell and T-cell **(Figure 1)**. The symptoms of the disease include fatigue and exhaustion, nosebleeds, weight loss, skin pallor, petechiae and infections.

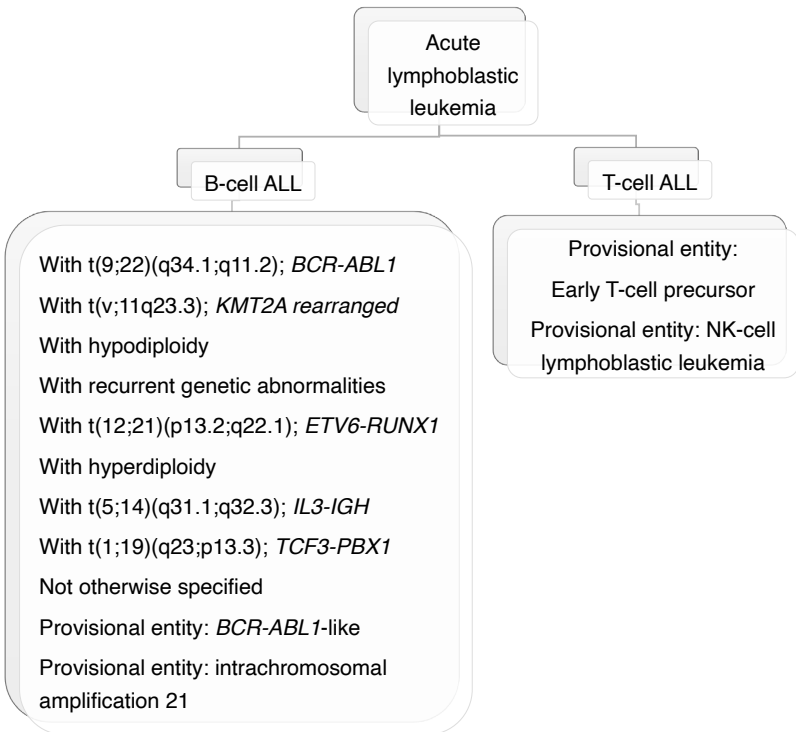

Figure 1. *WHO classification of acute lymphoblastic leukemia, edition 2016* [7]

According to the Swedish Childhood Cancer Foundation (Barncancerfonden), the combined share of leukemias and lymphomas accounts for about 42% of all cases of childhood cancers in Sweden. [8] Swedish National Board of Health and Welfare (Socialstyrelsen) provides a detailed statistical data on the incidence of ALL **(Figure 2A** and **2B)**. [6,9] The disease occurs mainly among children 0-4 years of age, affecting boys over girls, and shows a remarkable incidence stability over the years. [10]

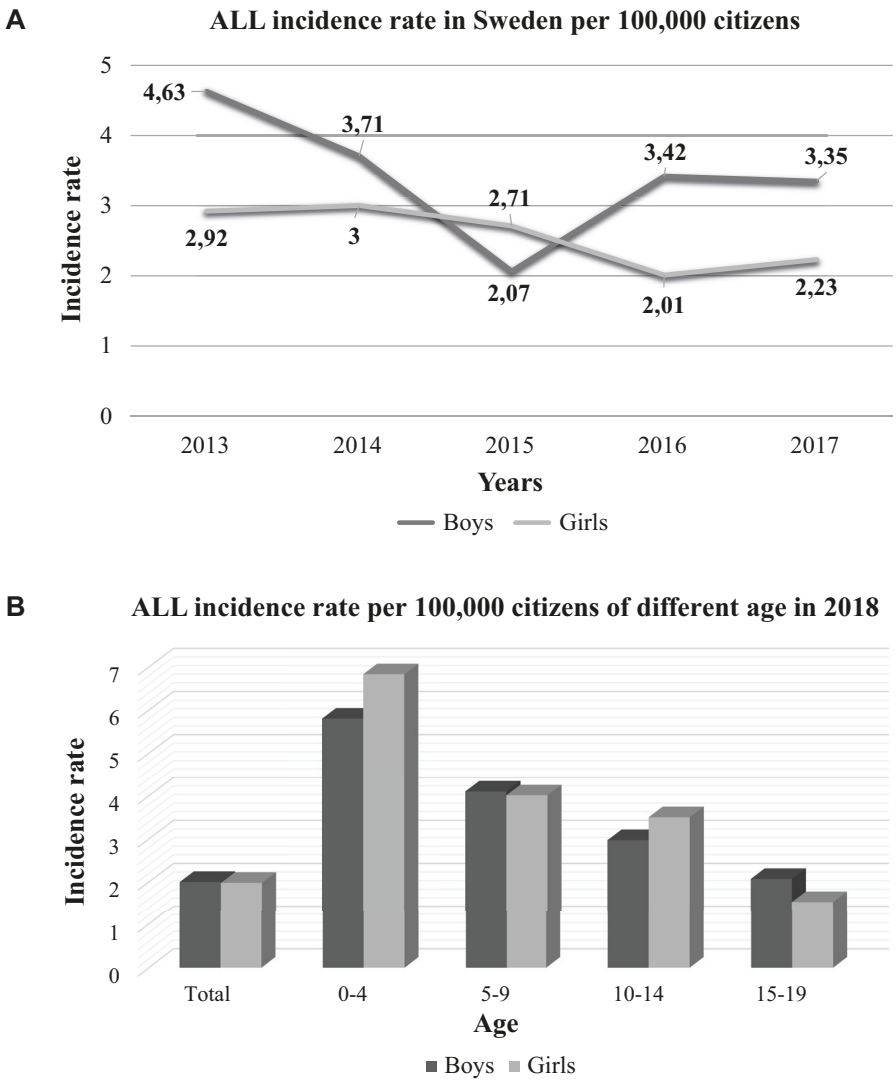

Figure 2. *Incidence rate ALL among Swedish children 0-19 years old according to Socialstyrelsen statistical database.[9,11] (A). Incidence rate of ALL (ICD-7) in Sweden in 2018 in different age groups (B).*

1.2.1 Mutational profile

Acute leukemia is a hematological malignancy that arises from a so-called leukemia-initiating cell. [12] This cell accumulates prenatal and postnatal mutations, and undergoes clonal expansion, resulting in leukemia. The clinical picture develops when a high number of mutated lymphoblasts suppresses the cell growth of other hematopoietic cell lineages. Possibly, after treatment the primary clone undergoes clonal evolution and may become the cause of a relapse. In pediatric T-cell and B-cell ALL lymphoblasts express different surface antigens, called cluster differentiation (CD) and are identified as immunophenotypic markers of the cell maturation. [13,14] In the clinics, these markers are important for the future diagnostics and treatment.

T-cell and B-cell ALL mutational profile includes more than 100 genes with studied aberrations, and some of them function as targets for treatment and/or prognostic markers. [15–18] The most common alterations in T-cell ALL are *NOTCH1* mutations, cyclin-dependent kinase inhibitors 2A and 2B (*CDKN2A/2B*), *TAL1* and Mixed Lineage Leukemia (*MLL*) gene rearrangements [15] **(Figures 1 and 3).** For B-cell ALL, *EVT6-RUNX1* (known also as *TEL/AML1*), *BCR-ABL1* (Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1) and *BCR-ABL1*-like mutations, together with *MLL* rearrangement are the most common genetic aberrations. [19] *BCR-ABL1 is a* fusion gene, or Philadelphia chromosome (Ph^+), that occurs in approximately 10% of B-cell ALL and hypodiploidy are associated with unfavorable prognosis. [17] In contrast, hyperdiplody, an increase in the number of chromosomes, occurs approximately in 30% of B-cell ALL and is an indicator of favorable prognosis in the patients.

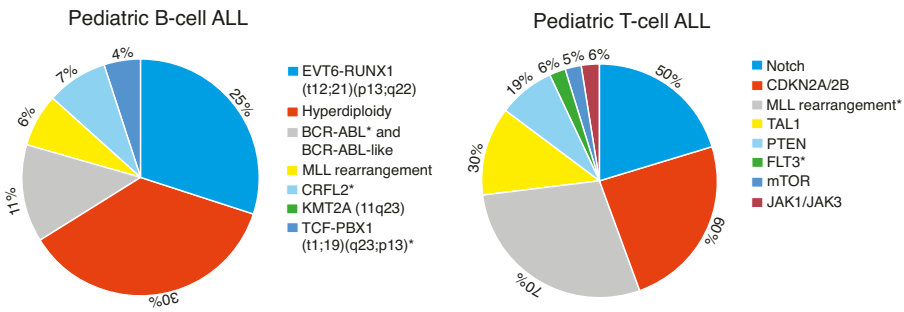

Figure 3. *The most common cytogenetic aberrations observed in children with B-cell ALL and T-cell ALL, Goroshchuk et al., Oncogene, 2019* [15]

U.S Food & Drug Administration (FDA) and European Medicines Agency (EMA) are responsible for the approval of the newly developed drugs in the USA and European Union member states. On the FDA website there is a list of relevant pediatric molecular targets for different types of tumors, including T-cell and B-cell ALL. [20] Overall, the potential treatment targets are the groups of tyrosine or serine/threonine protein kinases, cell surface receptors, transcription factors, enzymes, or cytokines, that have been identified as influencers on cancer development and prognosis.

1.2.2 A historical perspective of treatment

Despite the progress in diagnostics in the 19th century, there had been no effective treatment for acute leukemia for many decades. Nonetheless, accumulation of clinical reports on leukemia cases allowed to proceed with better understanding of the disease mechanisms that would lead to the development of a possible treatment. Important steps were made after the discovery of the blood groups made by Karl Landsteiner in 1901.[1] In 1947 Bessis and Bernard performed complete blood transfusion and achieved a short-term remission in a patient with leukemia. Blood transfusions were also widely used in children to compensate for severe hemorrhages. Also, physicians used potassium arsenite, mustard gas, busulphan and folic acid antagonists to treat leukemia. It was shown that these substances have a toxic effect on the hematopoietic system and can therefore eradicate the leukemic blasts.

By 1960s, there were several chemotherapeutical agents used to treat leukemia patients that were approved by FDA, such as adrenal corticosteroids and adrenocorticotrophic hormone; purine analog 6-mercaptopurine (6-MP); folic acid antagonist methotrexate (MXT); vincristine and cyclophosphamide. However, most of the drugs showed only temporary efficiency and many patients relapsed after treatment. The revolution happened when Donald Pinkel, an American doctor from St. Jude Hospital became inspired by multi-drug combinations in treatment of tuberculosis and Hodgkin disease. [21] He wrote a new protocol of intensive treatment with “armamentarium” of existing antileukemic drugs “The Total Therapy V Study”, that also included irradiation of the spine and cranium. In 1968 Pinkel and colleagues published a revolutionary paper reporting the long-term remission and high survival rate in children with leukemia. [22] This paper completely changed the approach in the treatment of childhood leukemia. It provided a basis for further improvements and protocol optimizations by different collaborative projects: Acute Leukemia Group B, Nordic Pediatric Hematology Oncology Group, Dutch Childhood Oncology Group, Berlin Frankfurt Munster Group and others. [21,23] Further studies resulted in stratifying patients into different risk groups, and understanding the role of genetic and chromosomal alterations in the disease prognosis. [23]

After years of work in hematology-oncology, Donald Pinkel identified four main obstacles in treating acute leukemia, such as (1) primary and secondary drug resistance; (2) isolated relapse in central nervous system; (3) high and overlying toxicities of existing drugs; (4) desperation to find the treatment. [21] More than 50 years later, his statements are still valid, except the one – desperation to find the treatment. There are many questions on how to help the patients waiting to be answered, and this thesis is one of the attempts to find a novel treatment for acute pediatric leukemia and improve long-term outcomes in patients.

1.2.3 Risk stratification

The development of the first chemotherapeutic protocol in the USA was followed by the rise of multiple national programs to treat pediatric ALL. In Sweden, Denmark, Finland, Norway, and Iceland, hematologists and oncologists apply protocols from the Nordic Society of Pediatric Haematology and Oncology (NOPHO). With one of them, NOPHO ALL-2000, it was possible to achieve a relatively long period of complete remission (CR), 50% 5-year overall survival (OS) and event-free survival (EFS). [24] In 2008 NOPHO set an aim to improve therapeutic strategy in patients with T-cell ALL and Philadelphia negative (Ph^-) B-cell ALL. Ten years, later in 2018 there were published the results on response to treatment and survival rates. [25] According to NOPHO ALL-2008 protocol, at first patients underwent stratifications at three time points: at diagnosis, 1 month and 3 months after diagnosis. Children were stratified into three risk groups: standard, intermediate, and high-risk. At the final stratification at 3 months after the diagnosis, approximately 50% of patients were included in a standard risk group as they satisfied the following criteria:

- B – cell lineage;
- white blood cells count (WBC) < 100 cells;
- minimal residual disease (MRD) < 10^{-3} day 29;
- no intermediate or high risk criteria/cytogenetics;
- no central nervous system 3 involvement at diagnosis.

Intermediate and high-risk groups included approximately 45% of patients. Stratification criteria included a particular cytogenetic profile, i.e. hypodiploidy or *MLL*-rearrangement, presence of blasts in cerebrospinal fluid, and MRD levels. [26]

1.2.4 Treatment strategy. Personalized therapy

The protocol NOPHO ALL-2008 includes different combinations of drugs based on the risk profile of each patient. The ALL treatment is divided into three phases: induction, consolidation and maintenance. [25,26] The induction phase of standard

risk patients includes treatment with one of the glucocorticoids (dexamethasone or prednisolone), vincristine, MTX, cytarabine, doxorubicin, pegylated (PEG)-asparaginase and 6-MP. In case of central nervous system involvement, intrathecal treatment is prescribed. The consolidation protocol includes all abovementioned drugs except doxorubicin and dexamethasone. Maintenance therapy phase of standard risk group includes the drugs from induction phase (excluding doxorubicin) with additional considerations of folinic acid and carboxypeptidase. [25] The expected duration of the treatment according to the protocol is 2.5 years, however, it always depends on the patient response. [26]

The chemotherapeutical protocol aims to attack the cancer cell by targeting different cellular processes. Dexamethasone and prednisolone suppress cytokine production and induce a specific glucocorticosteroid-mediated cell cycle arrest and apoptosis. [26,27] Vincristine belongs to a group of vinca alkaloids that destabilize the microtubule system of cancer cells and act as antimitotic drugs. Doxorubicin and daunorubicin belong to a group of anti-tumor antibiotics – anthracyclines. They induce DNA-damage via intercalation the DNA or inhibition of the enzyme topoisomerase II, also causing oxidative stress, irreversible disruption of mitochondrial functions and deregulation of iron homeostasis. [28,29]

The chemical structure of cytarabine is similar to nucleotide cytosine, therefore the drug incorporates into the DNA as a false base, leading to DNA synthesis inhibition in cancer cells. MTX is another drug that disrupts purine synthesis, it belongs to a group of antifolates. [30] Folic acid is an important part of the cell metabolism. MTX chemical structure is similar to the one of folic acid, and the drug competitively inhibits the enzyme involved in folic acid metabolism. This inhibition leads to a reduction in DNA synthesis and disturbed DNA methylation, followed by apoptosis.

Asparaginase catalyzes metabolism of a nonessential amino acid L-asparagine. [31,32] In case of leukemia, L-asparagine cannot be synthesized by cancer cells, therefore, they are dependent on the external sources of asparagine. Fast reduction of asparagine levels in blood, before the amino acid enters the cells, results in disrupted asparagine-dependent protein synthesis and a consequent cell death.

The last decades were enlightened by new successful drug developments. [33–35] FDA approved tyrosine kinase inhibitors (TKIs) imatinib and other *BCR-ABL1* inhibitors in patients with Philadelphia chromosome-positive (Ph+) ALL. TKIs are designed to bind to a specific region in ATP-domain, block the function of the mutated protein kinase and inhibit cancer cell proliferation. [36] These drugs significantly improved survival of the patients. [37,38] Immunotherapy is another exciting field that acts on activating the immune cells that were previously "tricked" and "blinded" by cancer

cells and cancer microenvironment. Natural killer (NK) cells targeted therapies, anti – cancer vaccines monoclonal antibodies and chimeric antigen receptor (CAR) T-cells are in the line of clinical trials and approved treatments. [33,39–41]

Overall, the approved intensive antileukemic treatment is efficient in eradicating of the lymphoblasts. However, short and long-term adverse reactions are important drawbacks of existing anticancer treatments.

1.2.5 Adverse effects of the current therapies

Even though the treatment is efficient towards cancer, [24] it causes multiple unwanted side effects by affecting normal cells. For example, folate receptors are expressed on both cancerous and non-cancerous cells, limiting the use of MTX. The severity of toxicity might be of a low grade and transient; however, sometimes the adverse reactions can lead to a lethal outcome. For example, chemotherapy and targeted drugs have a potential to induce tumor lysis syndrome that develops in case of intense cell proliferation and high tumor burden which is common in patients with hematological malignancies. [42,43] This serious condition can manifest first by changes in blood or changes in biochemical parameters, and later – with clinical symptoms that require an immediate and intensive treatment. The symptoms include high fever, renal and cardiac failure, and vary in severity, often leading to mortality.

Among the common side effects of corticosteroids are changes in the body weight, hypertension, immunosuppression with consequent increase of infection risk. [44] Due to the decreased cytokine production, patients have less pronounced clinical picture of life-threatening inflammatory processes. Additionally, treatment with steroids can induce withdrawal syndrome with plethora of symptoms from high fever and weakness to anorexia and mood changes.

As mild reactions, vincristine can provoke loss of reflexes, bone pain and/or constipation. [26] However, it can also lead to severe neuropathy. Topoisomerase II inhibitors, such as doxorubicin or etoposide induce multiple side effects such as BM suppression, hair loss, gastro-intestinal tract damage, reduced muscle strength and cardiotoxicity, including cardiomyopathy and irreparable heart failure. [45] Moreover, treatment with anthracyclines is associated with an increased risk of the secondary myeloid leukemia. [6] PEG-asparaginase can induce a specific allergic reaction with local redness and rashes, pancreatitis, thromboembolic complications, hyperglycemia or hyperbilirubinemia. [32] In severe cases PEG-asparaginase induces anaphylaxis. Cytarabine, one of the most used drugs in pediatric ALL, provokes multiple side effects, which are often dose-limiting factors. Among them are leukopenia, thrombocytopenia, rashes, fever, gastrointestinal inflammation, personality changes and neuropathy. [26]

Immunotherapy, including anti-CD19/CD22 CAR T-cells treatment became one of the highlights in cancer therapy in recent years. [33,46] However, this type of treatment is associated with a severe condition called cytokine-release syndrome. [47] An uncontrolled release of interferon gamma (IFNγ) and interleukins (IL), IL-6 in particular, results in systemic response including high fever, neurologic, respiratory, gastrointestinal and hepatic symptoms, and requires intensive monitoring and care. [48,49]

Small molecules also face the problem of unpredicted toxicities. [50,51] One study showed that the crystal structure and presence of pseudosequences of a serine/threonine protein kinases increased their promiscuity to a specific compound/small molecule, that might result in unspecific effects. [52] For the group of TKIs some of the common adverse reactions were reported, such as myelosuppression, skin rash, fatigue, diarrhea, and hypertension. [53] It is also known that point mutations in the drug targets later lead to the drug resistance. [54] Notably, the small molecule inhibitors may affect other proteins and pathways due to structural similarities between the proteins. At the same time, there is a possibility of disruption of the protein or receptor function due to unspecific downstream effect, without applying a targeted inhibitor. [55] For example, *in vivo* studies suggested histone deacetylase inhibitors (HDACi) with a monoclonal antibody to be a promising combination for myelodysplastic syndrome (NCT02936752). [56] HDACi inhibit expression of PD-L1 surface proteins, important for the tumor surveillance, and therefore, enhance the effect of monoclonal antibodies. However, the same effect of HDAC surface proteins degradation is observed by combining the antifolate pemetrexed and phosphodiesterase inhibitor sildenafil, despite that they do not aim to inhibit HDAC.

To conclude, there is a need for more knowledge in the "on-target" and "off-target" interactions, that might be used in the future to optimize treatment protocols and reduce adverse effects in patients.

1.3 Polo-like kinase family

In 1970s-1980s the scientists Nurse, Hartwell and Hunt described the cell cycle initiation and progression via genes responsible for checkpoints between the phases and characterized the key regulators of the cell division – cyclins and cyclin-dependent kinases (CDKs). In 2001 they were awarded the Nobel Prize for their discoveries. [57] Later it was shown that there are many more proteins that orchestrate the process of cell division. [58–60] The cell cycle of eukaryotic, including human cells, consists of interphase and mitosis. Interphase includes G1, S and G2 phases, and is required for the DNA replication and preparation for mitotic division. [58] Mitosis is divided into four phases (prophase, metaphase, anaphase,

telophase) and results in the formation of two cells via cytokinesis. The transition between the different phases is supported by checkpoints that ensure correct execution of all steps in the cell cycle.

The Nobel Prize press-release in 2001 stated that the knowledge about the regulators of the cell cycle can be applied for cancer diagnostics, and in a long-term perspective – for cancer treatment. [57] In 2019 more than 7 000 trials targeting cell cycle proteins in various diseases, from degenerative disorders to cancer, were registered by the U.S. National Library of Medicine. [61] The available drugs target not only cyclin-dependent kinases (CDKs) and cyclins, but also a number of other cell division regulators, including polo-like kinases (**Figure 4**).

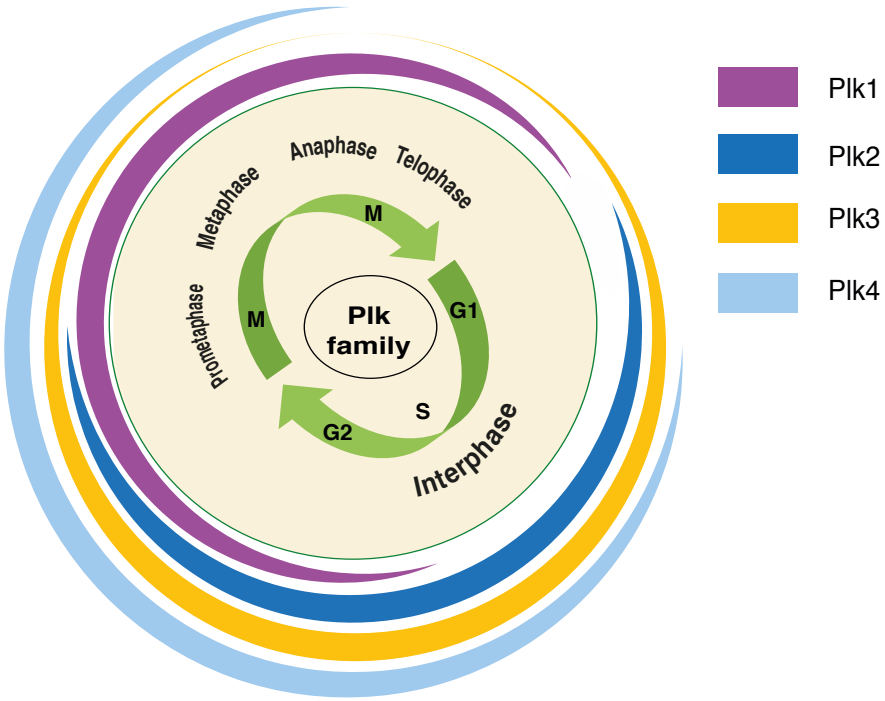

Figure 4. *Schematic description of the functions of different polo-like kinases in the cell cycle. The wider the line, the higher is the protein level of a particular kinase. Reprinted from Goroshchuk et al., Oncogene, 2019* [15]

In 1980s, experiments in Drosophila melanogaster showed that mutations in the *polo* gene disrupted the function and structure of centrosomes and induced abnormalities in mitotic spindles formation during the mitotic process. [62] In humans the discovered protein was called polo-like kinase due to a structural similarity with the protein encoded by *polo* gene. [63] Later it has been shown that the structure of

different Plk family members **(Figure 5)** is conserved throughout many species. The N-terminal in all Plks has an ATP-binding kinase domain that is one of the main targets for drugs, while the C-terminal contains a polo-box domain (PBD) which is a unique feature of the Plk family. [64,65] The Plk family proteins were identified both in cytoplasm and nucleus during the different cell cycle phases. Translocation to the nucleus is regulated by nuclear localization signal (NLS) that has already been identified on Plk1 (black mark in **Figure 5**) [66], however, more studies are needed to confirm the NLS sequences in Plk family members (yellow marks in **Figure 5**). [67]

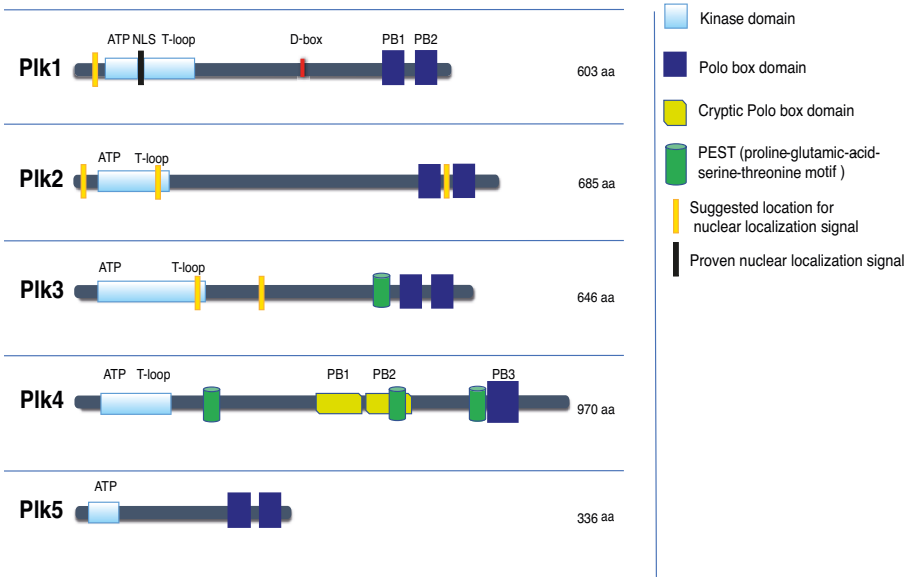

Figure 5. *The structure of all five Plk family members includes conserved serine/threonine kinase (N-terminal) domain and a conserved domain with two or more polo boxes (C-terminal domain). Modified from Goroshchuk et al., Oncogene, 2019* [15]

1.3.1 Polo-like kinase 1

Plk1 is the most studied kinase among all the family members. The transcription of Plk1 is regulated according to the process of cell division. There are two key factors, that coordinate the cell cycle: retinoblastoma (*Rb*) gene and *E2F*. While *Rb* suppresses the *Plk1* promotor [68,69], members of the E2F family, *E2F1-E2F3* activate transcription of *Plk1*. [70] The levels of *Plk1* messenger RNA (mRNA) are cell-type dependent and change during the cell cycle progression. The highest expression of *Plk1* is detected in BM, placenta, liver, pancreas, thymus, and testis, supporting the idea that Plk1 is important for mitotically-active cells **(Figure 6)**. [71,72]

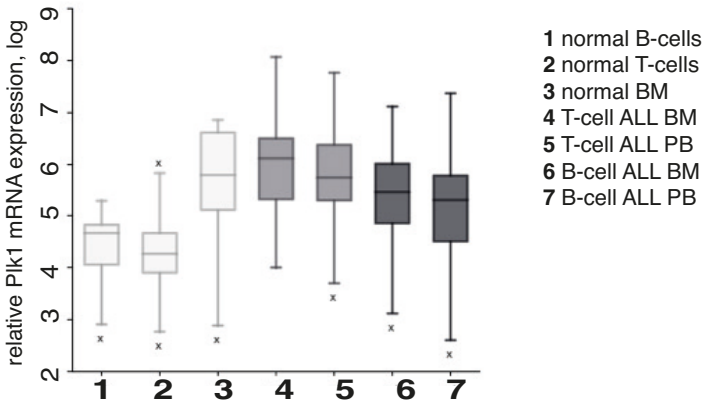

Figure 6. *Plk1 expression data from 477 samples uploaded to the publicly available databases, indicating that Plk1 is highly expressed in actively dividing cells, i.e. in bone marrow (BM) or malignant lymphoblasts in peripheral blood (PB)*

Before the G2 phase, Plk1 is detected in cytoplasm when the kinase domain is autoinhibited by the PBD, while the active phosphorylated form of Plk1 is located in the nucleus. [73–75] There are two main active sites on the N-terminal of Plk1 protein, Ser137 and Thr210; in autoinhibited state Ser137 forms a hydrogen bond with Glu126 from the PBD. Ser137 and Thr210 phosphorylation relieves this bond and activates the kinase. [75,76] Plk1 appears at multiple events during the cell cycle **(Figure 4)**. Despite that initially the role of Plk1 was shown in G2 and mitosis progression, more experimental evidence points out that Plk1 is important in G1 and S phases. For example, proper Plk1 degradation via ubiquitination is important for G1/S progression. [77] Plk1 becomes active after phosphorylation on Thr210 by Aurora kinase A and its cofactor Aurora Borealis (Bora). The phosphorylated Plk is then recruited to the nucleus during the G2 phase. [76,78] There Plk1 regulates centrosome maturation and correct microtubule – kinetochore attachment. [79,80] Plk1 ensures correct G2/M transition due to the interplay between Plk1 and complex Cdk1-cyclin B1. [59] Before the M-phase, Cdk1-cyclin B1 is inhibited by kinases Wee1 and Myelin-transcription factor 1 (Myt1). Plk1 inhibits Myt1 and promotes Wee1 degradation [81], together with phosphorylation and activation of Cdc25C phosphatase that dephosphorylates Cdk1 and promotes enter to mitosis. [82] The process of cleavage furrow formation during cytokinesis is also regulated by Plk1. In this case, Plk1 phosphorylates proteins Protein regulator of cytokinesis 1 (PRC1), Kinesin family member 23 (KIF23) and RACGAP1 (Rac GTPase activating protein 1) that activate enzyme Ras homolog family A (RhoA) and ensure separation of the two cells. [59,83] When Plk1 has completed its functions,

it is ubiquitylated and degraded by Anaphase-promoting complex/cyclosome$^{\text{Cadherin 1}}$ (APC/C$^{\text{Cdh1}}$) at the end of mitosis. [65,84,85] Nondegraded Plk1 causes a reduction in APC/C$^{\text{Cdh1}}$ activity, a defect in the destruction of mitotic cyclins, and a delay in mitotic exit. Furthermore, recent studies also revealed functions of Plk1 that are not connected to the cell cycle: Plk1 regulates vascular homeostasis and supports response to angiotensin II in cardiomyocytes. [86]

Not only normal cell cycle, but the stress response mechanisms and malignant transformation are dependent on Plk1 as well. In 1996 Lane and Nigg injected highly specific anti-Plk1 antibodies in HeLa cells in order to investigate the functional role of Plk1 inhibition in cancer cells. [87] The injection resulted in abnormal distribution of chromatin, unseparated centrosomes, and overall failure in the cell division. It has been also shown that Plk1 is involved in centriole reduplication and maturation process during the S-phase in malignant cells. [88] In case of DNA damage, Plk1 is one of key targets of DNA damage response signaling cascade. The interplay between synthesis and degradation of APC/C and Cdk1-cyclin B1 complexes supports the one-way progression of the normal cell cycle. In case of early DNA damage, Plk1 phosphorylation is prevented by Aurora A inhibition; while later induction of DNA damage signaling in G2 phase leads to Plk1 degradation by APC/C$^{\text{Cdh1}}$. However, in turn, Plk1 can inhibit DNA damage mechanisms response and promotes the checkpoint recovery via inhibition of Wee1, ataxia-telangiectasia and Rad3-related protein (ATR)-Checkpoint (Chk) 1, ataxia-telangiectasia mutated (ATM)-Chk2, and p53 axes. Several studies showed the role of Plk1 in a complex interplay with p53 in normal and cancer cells. [89–91] Plk1 either phosphorylates E3 ubiquitin-protein ligase TOPORS and G2 and S phase-expressed protein 1 (GTSE1) that activate p53 suppressor E3 ligase MDM2, or directly phosphorylates p53, leading to its inhibition. In turn, p53 binds to Plk1 promoter region and directly inhibits Plk1 expression, regulating mitotic progression. [89] Overall, it has been shown that without Plk1 cancer cells are unable to continue cell division and Plk1 inhibition induces mitotic catastrophe after checkpoint disruptions or DNA damage. [81,92]

Since Plk1 is involved in mitosis, the protein has been extensively studied in relation to cancer. RNA sequencing data combined with survival analysis showed that Plk1 upregulation has tumor-dependent positive, negative or neutral influence on prognosis in cancer patients. [93] For example, there are multiple studies that reveal an oncogenic role of Plk1 that might contribute to a poor prognosis in case of lung adenocarcinoma, gastric, bladder or kidney clear cell carcinoma patients. [71,72,93–99] At the same time, no effect on survival was observed in patients with ovarian cancer or cervical squamous cell carcinoma. According to the data analysis of the TARGET ALL and TCGA Acute Myeloid Leukemia (AML) studies in XENA browser, there is no significant connection between the level of *Plk1* expression and survival of the patients. [100]

1.3.2 Polo-like kinase 2

Unlike Plk1 that is expressed in dividing cells, Plk2 is ubiquitously expressed throughout human tissues. [59] *Plk2*-/- fibroblasts and embryos are viable, although they demonstrate an increased duplication time and slow growth. Both mRNA and protein levels of Plk2 peak during the G1 and early S-phases. In normal cells, Plk2 is involved in centriole duplication during G1/S transition.[59,101]

The potential role of Plk2 in cancer has also been studied. Plk2 CpG islands are epigenetically silenced in a number of hematologic malignancies (e.g., Burkitt lymphoma, AML, myelodysplastic syndrome (MDS), multiple myeloma) suggesting the tumor suppressing role of Plk2. [102] It was also shown that higher Plk2 CpG methylation levels correlated with a more resistant cancer cell phenotype elucidating potential involvement of Plk2 in drug sensitivity and therapy response. [102,103] However, some studies showed that Plk2 phosphorylates mutated p53. In this case Plk2 knockdown might be used to combat chemo-resistance in p53-mutated tumors. [104]

1.3.3 Polo-like kinase 3

The biological role of Plk3 in different types of cells is disputable. [59] In general, it has been shown that Plk3 is involved in the cell cycle by regulating G1/S transition. [67] At the same time, it has been shown that Plk3 phosphorylates the anti-apoptotic protein Bcl-xL at G2/M checkpoint and promotes G2 arrest. [105] During the DNA damage and stress response Plk3 is able to phosphorylate p53. [106] In malignancies, apart from expression data showing *Plk3* loss in several cancer types, [107] it was demonstrated that Plk3$^{-/-}$ mice exhibited enhanced angiogenesis and were prone to tumor development at older age. [108] Recently it has been found that Plk3 inhibits the activity of an oncogene hypoxia-induced gene (HIF1a) known to promote angiogenesis and glucose metabolism in cancer. [109,110] Therefore Plk3 has been proposed to play a tumor suppressor role [107,111]

1.3.4 Polo-like kinase 4

Plk4 protein structure is similar to other Plk family members, however, the C-terminal part contains a unique structure called the cryptic PBD (**Figure 5**). [84,112] Due to this difference, the regulation of Plk4 activity is distinct from that of Plk1-3 which utilize PBD dimerization for autoregulatory functions. Plk4 mRNA and protein expression is low during G1- and S-phase and increases during mitosis. It has been shown that Plk4 deficient fibroblasts cannot progress and die early in the development. [59] [113] The death at one week of embryonal development followed a complete *Plk4* gene knockout, while heterozygous mice develop cancer

in the older age, having an increased level of cyclin B1 and multiple spindles in the cancer cells. Plk4 is almost exclusively localized on centrosomes, and is involved in centriole and basal bodies duplication during the S-phase. [114–116] As observed in overexpression experiments, increased levels of Plk4 lead to tri- and tetrapolar spindles formation. [115]

The role of Plk4 in cancer is also being extensively studied since Plk4 is frequently overexpressed in cancer cells. Some studies showed that Plk4 inhibition leads to anti-cancer therapy sensitization by limiting the replicating ability of cancer cells. [117] It was also found that Plk4 has centrosome-independent functions that promote cancer cell migration and invasion. [118] As an increased expression of Plk4 promotes chromosomal instability, the protein was qualified as a desirable target for anti-cancer drug development. [119,120] Currently, phase I clinical trial is recruiting patients with a relapsed or refractory AML for treatment with Plk4 inhibitor CFI-4000945 fumarate (NCT03187288). [120]

1.3.5 Polo-like kinase 5

The recently discovered Plk5 is located in nucleolus and is present in neuronal and reproductive tissues in vertebrates. [121,122] Human Plk5 has a stop codon at exon 6 that leads to synthesis of truncated human Plk5 protein. [59] The mechanism of action of Plk5 is different from other Plk family members, for example, the stress-response mechanism in Plk5 is independent of p53. [121] In neurons and glia, Plk5 is required for neurites growth. [122] In humans, an increased Plk5 promoter methylation is observed in aggressive astrocytomas, suggesting Plk5 as a treatment target in brain tumors. [121,123]

1.4 Plk1 inhibition

Since Plk1 is highly expressed in different cancer types, Plk1 has become an attractive target for anti-tumor drug development. To date, several approaches have been tested. For example, small molecule inhibitors bind to different domains in already synthesized Plk1 protein, while antisense oligonucleotides (ASOs) and short interfering RNA (siRNA)-based drugs aim to induce *Plk1* mRNA cleavage. Each of these treatments will be discussed below.

ASOs are single-stranded “gapmers” with “gaps” of several DNA nucleotides inserted between chemically modified RNA nucleotides. [124] ASOs enter the cell without being conjugated to a specific delivery domain in order to cross the cell membrane bilayer. [125] The enzyme RNaseH identifies and recruits to DNA:RNA duplexes, inducing the cleavage of the target mRNA. [126] Several attempts to apply

such strategy to inhibit Plk1 showed that antisense inhibition of *Plk1* mRNA using phosphorothioate-modified ASOs led to loss of cell viability and proliferation arrest. [127,128] The injection of ASOs lead to anti-tumor activity on the lung cancer cells in xenografted nude mice. However, the most promising treatment results with ASOs were reached in targeting liver and central nervous system. [129] As for the beginning of 2020, no clinical study was initiated to use ASOs in ALL treatment. In contrast to ASOs, small molecule protein inhibitors have been more successful in reaching the cancer patients in clinical trials.

1.4.1 Plk1 inhibition by small molecules

Among the important advantages of small molecules are high penetration through the cell membrane and a relatively inexpensive production. Plk1 has two "druggable" domains – the kinase domain with an ATP-pocket and the PDB, therefore many ATP-competitive or PBD inhibitors have been developed. However, PBD inhibitors developed up to date, such as poloxin or thymoquinone turned out to be non-specific alkylators. [130] At the same time, new papers have been published on synthetic PBD inhibitors, indicating an ongoing interest in this way of Plk1 inhibition. [131,132] However, there are no ongoing clinical trials on PBD inhibitors. In contrast, ATP-competitive Plk1 inhibitors that induce G2 arrest and trigger apoptosis in cancer cells have been analyzed in multiple past and ongoing clinical trials. [15,72,133–135]

One of the earliest ATP-competitive inhibitors is BI2536. It belongs to a group of dihydropteridinones and induces prometaphase arrest with the formation of monopolar spindles, mitotic disruption and a cell apoptosis in multiple cancer cell lines. [136–138] The IC_{50} value of BI2536 is 0.83 nM, 3.5 nM, and 9.0 nM towards Plk1, Plk2, and Plk3, respectively. [139] In successful trials 23% of the patients had a complete remission (CR) for longer than 3 months. The main dose-limiting toxicities (DLTs) reported were reversible neutropenia with or without infection, nausea, fatigue and anorexia of mild to moderate intensity (**Figure 7**). [61] Despite the met endpoints and antitumor effect of BI2536 in patients with metastatic tumors or myeloid leukemia, BI2536 did not go beyond phase II/IIa of clinical trials.

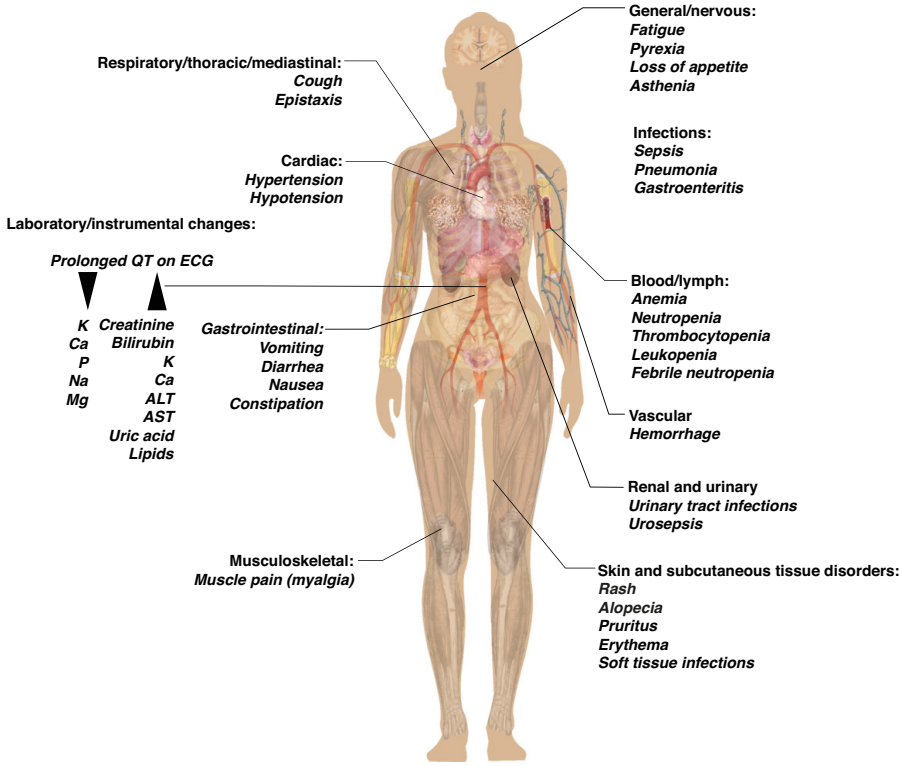

Figure 7. *The most common side effects, reported from clinical trials with Plk1 small molecule inhibitors BI2536, volasertib and NMS-1286937 as single drugs. Enrolled patients had solid tumors and hematological cancers. One study (NCT01971476) was conducted in children. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ECG, electrocardiogram; QT, interval that describes contraction and relaxation of cardiac ventricles*

After BI2536 followed another dihydropteridinone derivative and ATP-competitor named volasertib (BI6727). Volasertib was shown to be an efficient Plk1 inhibitor, with an IC_{50} value of 0.87 nM. However, due to the structural similarity of the ATP-pocket, volasertib also inhibits Plk2 and Plk3 (IC_{50} values of 5 nM and 56 nM respectively). [140] Volasertib has been tested in multiple pre-clinical trials, both as a single agent and in a combination therapy and showed a potential for the treatment of resistant forms of leukemia. [138,141–144] Volasertib has also been examined in several phase I-II clinical trials and initially showed acceptable tolerability and safety for patients, with no increase in death rate. [145,146] Most commonly, the effects of volasertib in patients were studied in combination with other drugs, such as cytarabine, azacytidine or the histone deacetylase inhibitor belinostat. [147–151]

However, some studies were withdrawn or terminated before achieving the main objectives, or co-administration of volasertib with abovementioned drugs did not improve survival of the patients. [152] In phase III trials POLO-AML-2 combination with cytarabine showed a higher incidence of neutropenic fever and fatal infections (**Figure 7**). [153]

The third Plk1 small molecule inhibitor, NMS-1289637 (NMS-P937, onvansertib) has been developed recently. [154–156] NMS-1289637 specifically and reversibly inhibits Plk1 at an IC_{50} value of 2 nM, showing activity in multiple cell lines, with a minimal effect on Plk2 and Plk3. [155] After promising *in vivo* experiments, the possibility of an oral formulation, beneficial pharmacokinetics and pharmacodynamics, and a proven antitumor effect led to the start of a phase I trial in adult patients with metastatic and advanced solid tumors. [157] As major dose-limiting toxicities (DLTs) reversible anemia, thrombocytopenia, leukopenia and neutropenia were reported (**Figure 7**). Therefore, the drug was recommended for the future investigation and was recently enrolled in a phase II clinical trial on solid tumors and acute leukemia. [158]

In summary, the area of small molecule inhibitors is expanding as more pharmaceutical companies aim to use the ATP-pocket to block mitotic activity of Plk1.

1.4.2 RNA interference against Plk1

1.4.2.1 RNAi: from flowers to humans

The discovery of RNA interference (RNAi) began in 1990 with an experiment on the petunia flower, where the effect of RNAi was observed for the first time. [159] However, there was no clear explanation of the phenomenon. Later, in 1998 Craig Mello and Andrew Fire described and explained the process of RNAi in the nematode Caernorhabditis elegans (C.elegans). [160] They were awarded a Nobel Prize in Physiology and Medicine in 2006 for their studies.

The process of RNAi is very elegant and is based on the evolutionary developed response towards viral intrusion and is used to disrupt the expression of alien genes in cytoplasm. When a double-stranded (ds)RNA molecule enters the eukaryotic cell, it is cleaved by a specific enzyme Dicer into siRNAs (up to 21 nucleotides). Further, the double-strand RNA – binding protein (dsRBP) binds to the siRNA/Dicer complex and together with the enzyme Argonaute (Ago) 2 they form the RNA-induced silencing complex (RISC). In this complex, the passenger (sense) siRNA strand is removed, while the guide (antisense) strand is retained and is used as a template to identify the target mRNA that is cleaved by Ago2 in a sequence-specific manner. Consequently, the absence of the mRNA prevents the synthesis of the corresponding protein.

1.4.2.2 RNAi: from bench to bed

In oncology, the opportunity to modify mRNA expression opened the way to target genes crucial for cancer cells, i.e. responsible for the cell proliferation or migration. [161] However, the potential of siRNA-based therapies is still limited by different issues such as delivery inside a target cell, immune response, escape to the cytoplasm, and overcoming these issues has been a major task. [125,126] As the area of RNAi-based therapies is quickly expanding, several rules have been set on how to design siRNAs for *in vivo* studies and future clinical implementation. [126,162,163]

First, the size and the negative charge of siRNA prevents the passive diffusion across the bilayer cell membrane. The siRNA molecule is too large and too negatively charged for a passive diffusion, and in a modified state can be up to 70 times larger than a small molecule drug **(Figure 8)**. [126]

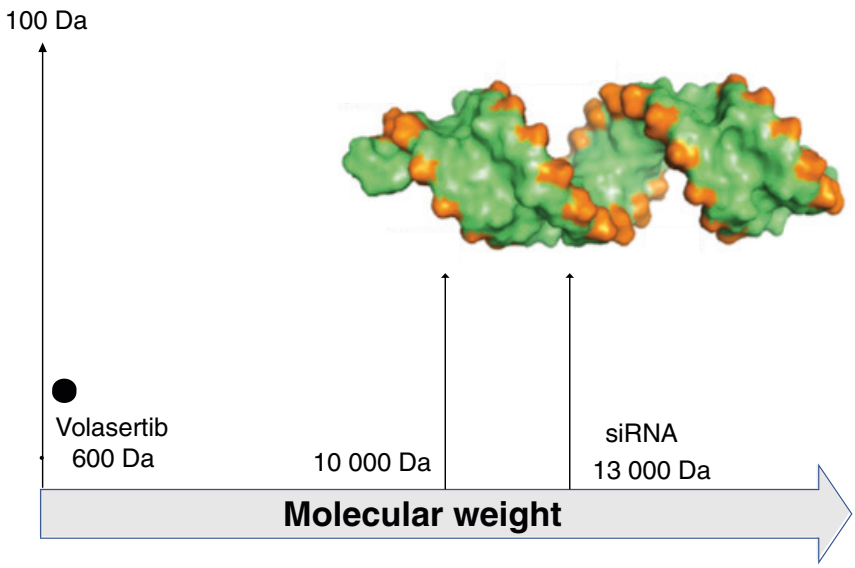

Figure 8. *Schematic comparison of molecular weight of the Plk1 small molecule inhibitor and siRNA.*

Therefore, multiple structures that can carry siRNA as cargo inside the cell have been developed. These include antibodies, cell-penetrating peptides, lipid or polymer nanoparticles, that can deliver the siRNA molecule into the cytoplasm via surface receptor-mediated endocytosis. [126,161,164,165] The issue with lipid nanoparticles arises from the complexity of the structure due to the involvement of up to five components. Each component has a different level of toxicity, that can induce unspecific

and adverse effects. Moreover, it is difficult to deliver the lipid nanoparticles to the cells other than hepatocytes. [125] In contrast, the clinically successful choice of the target and delivery domain was made with acyloglycoprotein receptors on hepatocytes and liver-targeting ligand tris N-acetylgalactosamine (GalNAc), that will be discussed later.

Second, upon the injection, the siRNA can be rapidly taken up and cleared from the blood by scavenger receptors on kidneys and the liver. Therefore, siRNA molecules have to be able to evade destruction by serum nucleases, have a long circulation time, escape renal clearance or opsonization. [165]

Third, throughout the human evolution, the cells have developed a tool to identify the alien RNAs called pattern recognition receptors, for example Toll-like receptors, dsRNA-dependent protein kinase (PKR) and retinoic acid inducible gene 1 protein (RIG1). [166] Toll-like receptor 3 (TLR3) is expressed on fibroblasts, epithelial and dendritic cells, and recognizes siRNA that interacts with the cell surface or inside the endosome. TLR7 and TLR8 are expressed on dendritic cells, B cells, monocytes and macrophages, and react on siRNA. Finally, PKR and RIG1 proteins detect siRNA in the cytoplasm. As a response mechanism, the immune cells produce IFNα/ß/γ and IL1/IL6/IL12. Therefore, in order to be able to execute its function *in vivo,* therapeutic siRNA should be synthetically masked from the pattern recognition receptors which are a part of the innate immunity. [163,164] Moreover, the siRNA sequence, the chemical structure and used delivery vehicles can also induce the immune response. [166]

Last but not the least, there is an issue of facilitation and controlling of the siRNA escape from the endosome. There are different types of endocytosis, such as phagocytosis, clathrin-dependent endocytosis, caveolae formation, micropinocytosis, but all of them exhibit an issue for siRNA delivery inside the cytoplasm in a non-toxic manner. [164] The exact mechanism underlying this process is still unknown. However it has been suggested that a pH drop inside the endosome could lead to endosomal membrane destabilization, and the experimental data confirm siRNA escape into the cytoplasm and induction of RNAi. [125,167,168]

The discovery of acyloglycoprotein receptors eligible for siRNA uptake on the hepatocytes surface resulted in an FDA-approved RNAi-based drugs. Patisiran ("Onpattro") was the first drug to be approved and is used to reduce transthyretin mRNA and treat transthyretin – mediated amyloidosis. Patisiran is encapsulated in lipid nanoparticles, coated with apolipoprotein E (ApoE), and after the intravenous injection it travels with the blood stream, gets to the liver and binds to the ApoE receptors on the liver surface. [49,169] The results of multiple clinical trials showed that patisiran induces mild infusion-related side effects such as back pain,

flushes, nausea, and/or abdominal pain. [170] Moreover, during the three – four years of clinical use only mild reactions were observed in some patients. Therefore, patisiran is considered to be a safe and efficient treatment. The second drug, givosiran, was evaluated in patients with an acute intermittent porphyria. [171,172] The drug is used subcutaneously and executes liver-targeting ligand GalNAc as a delivery vehicle for siRNA. GalNAc binds to acyloglycoprotein receptors and assists siRNA entry into a liver cell to reduce mRNA of delta aminolevulinic acid synthase 1 (*ALAS1)*. Among the most common reported adverse events in clinical trials were nasopharyngitis, abdominal pain, nausea, diarrhea, back pain, fatigue, headache, injection-site reaction, oropharyngeal pain, rash, and vomiting. [171] In general, adverse reactions were reversible of mild-to-moderate severity, therefore givosiran was approved by FDA in 2019 and entered the pharmaceutical market under the trade name "Givlaari". [172,173]

Notably, there were also attempts to target Plk1 using stable nucleic acid – lipid nanoparticles (SNALP) encapsulation as a delivery system for RNAi-based inhibitor TKM – 080301 (TKM-Plk1) in adult patients with hepatocellular carcinoma. [174] The study showed a good tolerability of treatment, with fever, fatigue, peripheral edema, noncardiac chest pain, and malaise as reported adverse reactions. Three cases of patients' death were considered as not related to TKM-Plk1 treatment. Despite this, due to a modest effect on tumor, TKM – Plk1 was not recommended as a single agent to treat hepatocellular carcinoma. As of the beginning of 2020, there have been no clinical studies initiated with siRNA Plk1 as a treatment for any kind of leukemia.

1.4.2.3 RNAi prodrugs

As it has been mentioned before, siRNA-based therapy has a great potential to treat cancer, and multiple research groups work to optimize the delivery approach for this type of drugs. One of the aims in this thesis was to evaluate the effect of modified siRNAs – RNAi-prodrugs, called short interfering ribonucleic neutrals (siRNNs) **(Figure 9)** in ALL. [167]

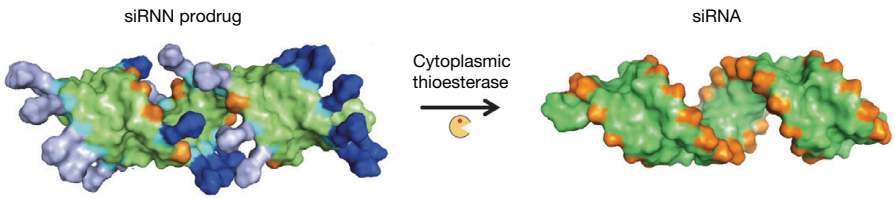

Figure 9. *siRNNs (left) with "masking" modifications, that turn into the "naked" siRNA (right) after entering the cell. Reprinted from Meade et al., Nature Biotechnology, 2014 with a kind permission from Springer Nature*

For the successful RISC formation, it is important to maintain the siRNA double-stranded helix shape, that severely restrict the possible modifications. The siRNN is a result of the several inventions in the nucleic acid chemistry that aimed to increase the success of *in vivo* drug delivery. For example, 2'- sugar modifications such as 2'-*O*-Me and 2'-F are some of the most commonly used modifications in clinical trials. They successfully mimic the original 2'-OH, but also help to escape the destruction by nucleases, to evade activation of the Toll-like receptors (TLRs) and induction of the interferon response in the blood. [166,175] It has also been shown that the 2'-sugar hydroxy group replaced by *O*-Me helps to avoid immune recognition by TLR7/8 without suppressing the capacity of RNAi. [166]

Further, the overall reduction of the siRNA charge promotes its stability and chances to be delivered to the target cell. Therefore, the negatively charged phosphodiester backbone of a naked siRNA should also be masked. For this reason, phosphorothioate, morpholino, phosphotriester and peptide nucleic acid modifications were proposed. [125] In our studies the phosphodiester backbone is modified with a neutral bioreversible phosphotriester group that is also used as a link to the delivery peptide. The modified drugs represent siRNAs that are conjugated to a TAT-peptide via an S-acyl-2-thioethyl (SATE) group that enables the delivery of siRNN into the cell. A TAT protein transduction domain, also known as a cell penetrating peptide, was suggested to cross the lipid bilayer of the cell membrane via endocytosis. [176] Upon entering the cytoplasm, the phosphotriester bonds are processed by cytoplasmic enzymes, named thioesterases which cleave the thioester bond within the SATE group. As the thioesterases are present only in the cytoplasm, the "de-masking" of the siRNN molecule takes place only inside the cell. The cleavage results in a two-step rearrangement process of the modified phosphotriester siRNN into a naked siRNA with a negatively charged phosphodiester backbone. Further, the siRNA is loaded into RISC together with Ago2, and the loading is followed by a targeted cleavage of Plk1 mRNA **(Figure 10)**.

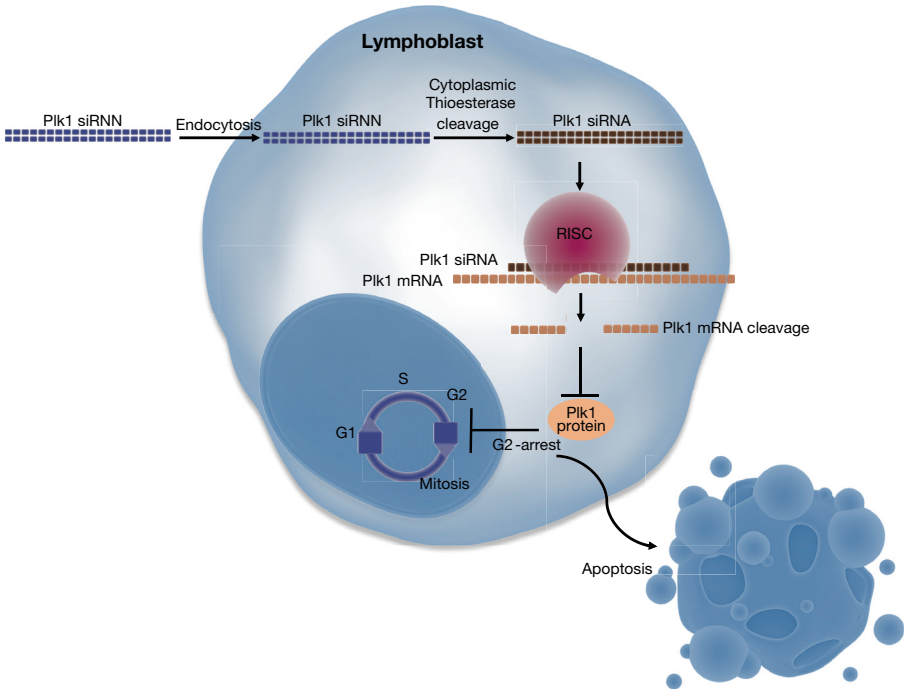

Figure 10. *Graphical description of Plk1 siRNN mode of action upon treatment.*

2 AIMS

The overall aim of the present thesis was to study the potential of Plk1 as a treatment target for pediatric acute leukemia using small molecule inhibitors and novel RNAi-based drugs.

Specific aims:

- To map off target proteins that are bound to the small molecule Plk1 inhibitors in order to shed light on the mechanism of adverse effects in patients treated with these drugs;
- To investigate the expression of the Plk family members in primary cells from pediatric ALL patients and healthy donors;
- To target Plk1 in leukemic blasts isolated from the PB and BM samples from T-cell ALL and B-cell ALL patients using siRNNs and a small molecule Plk1 inhibitor volasertib;
- To analyze the toxicity of siRNNs towards primary cells from healthy donors.

3 RESEARCH APPROACH AND MAIN FINDINGS

3.1 Paper I. Investigating the target landscape of Plk1 small molecule inhibitors using thermal proteome profiling

In this work, we applied a strategy to study the effect of Plk1 small molecule inhibitors on the multiple proteins based on the thermal stability of each protein. We used the results of thermal proteome profiling to get an insight into the mechanisms of the adverse effects found in patients treated in clinical trials.

Unpredicted adverse effects in patients enrolled in clinical trials on novel therapies is an important issue to be solved. Before a small molecule inhibitor reaches patients, it undergoes multiple preclinical studies *in vitro* and *in vivo*. During the drug development process the attention is paid mostly to confirm the target binding. Even preclinical studies on animals do not fully reveal the potential adverse effects that will occur in human patients. [55,177] Therefore, there is a need to investigate the off-targets of a drug in order to predict and potentially avoid adverse effects in the future.

Several methods have been developed to understand the drug-protein and protein-protein interactions under the different conditions. Multi-channel fluorescence microscopy was suggested to identify these interactions as a "live in-cell western blotting". [55] However, this method has its limitations, such as the necessity of blocking procedure or incubation with primary and secondary antibodies that require optimization due to potential issues with obtaining a strong and valid signal. Förster (also named fluorescence) resonance energy transfer (FRET) is another method used in studying the protein-protein interactions. FRET is used for screening of the small molecules binding to a one particular protein. [178] While the method requires designing of a specific biosensor for a studied protein, it limits the unbiased identification of previously unknown targets. [179]

We decided to use a different approach. It is known, that ligand-protein binding results in a conformational change followed by a shift in the protein melting temperature. The thermal shift – when detected – can potentially confirm a presence of a drug-protein bond (**Figure 11)**.

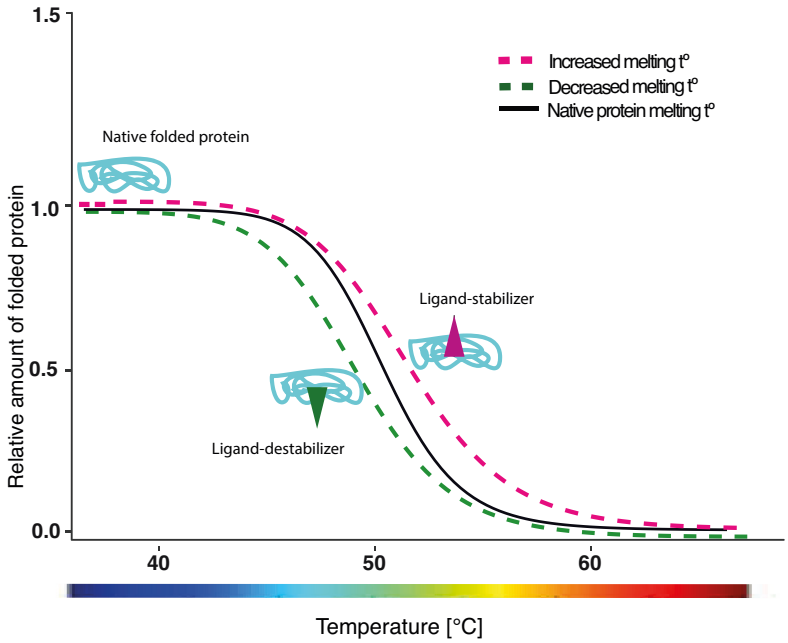

Figure 11. *Concept of the protein thermal shift upon drug treatment*

Cellular thermal shift assay (CETSA) is an affinity-based strategy that is used to evaluate the thermal stability of a drug-protein complex. [180] The method is applied for unbiased study of direct or downstream interactions in a complex cellular environment. The thermal shift can be detected by western blotting (WB) or liquid chromatography – mass spectrometry (LC-MS).

We used WB as a first step to confirm that Plk1 small molecule inhibitor volasertib bound to Plk1. We treated several cell lines to investigate if a melting shift is cell dependent. For the CETSA-WB the cells were treated, harvested, and lysed via triple snap freezing-thawing followed by centrifugation to separate cell debris from the cell lysate (**Figure 12**). [181] We confirmed that volasertib binds to Plk1 in different cancer cell lines by observing a thermal shift at 47 °C. Notably, this method relies on the availability of a specific high-quality monoclonal antibody against Plk1.

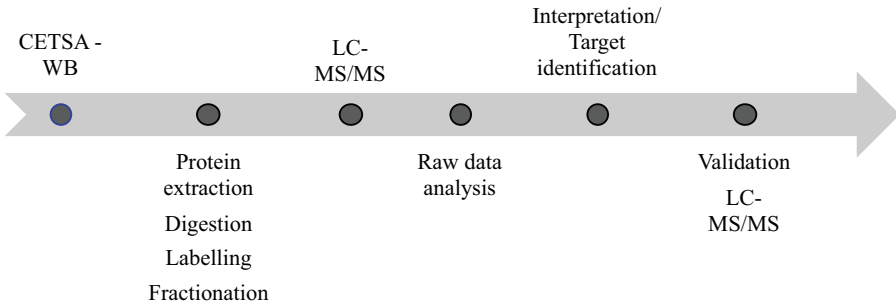

Figure 12. *Flow-chart of the experiments conducted for the paper I*

Thermal proteome profiling (TPP) is a development of CETSA when quantitative MS is used to identify binding between small molecule inhibitors and target/off-target proteins. [52,182] The use of this method led to discovery of the unexpected interactions by clustering the protein kinases in different groups according to the promiscuity to a specific compound. We investigated the proteomic profile of leukemic cells after treatment with three different Plk1 inhibitors namely BI2536, volasertib and NMS-1286937 by running Tandem Mass Tag 10Plex (TMT10Plex) for LC-MS/MS. TMT10plex is one of the variants of peptides labelling with 10 isotopic mass tags with further peptide separation during LC, fragmentation and quantification in MS/MS mode. For this experiment we treated Jurkat cells with 20 μM of the drugs. It is known that a high concentration of the drug decreases its selectivity. However, a micromolar-range dose was used to maximize the coverage of the proteins affected by the drug. Moreover, in preclinical mouse studies it was shown that treatment with 35 mg/kg of volasertib led to a drug accumulation in tissues with the highest concentration 32 μM at 8 h after treatment, compared to plasma concentration up to 7 μM after 1 h. [140] At the last time-point measurement, tumor and plasma concentration corresponded 4 μM and 8 nM respectively. This data supports the choice of 20 μM drugs concentration for TPP.

First, our results showed that BI2536 and NMS-1286937 also bind to Plk1 and change its melting temperature in the treated cells, compared to dimethyl sulfoxide (DMSO)-treated controls. Second, we obtained a list with several thousands of proteins that were detected by MS upon treatment. These hits were affected either by direct interaction with the drug or by downstream effect. We sorted the hits according to the non-parametric adjusted *p-value* for the further validation experiments, aiming to identify the relevant off-targets not compromising on specificity. [183] We compared the hit lists of the drugs and found no overlaps between three of them. Therefore, we decided to analyze the overlaps between the pairs "BI2536 – volasertib", "volasertib – NMS-1286937" and "BI2536 – NMS-1286937" using publicly available databases, such as STRING and ENRICHR. In the STRING

database, the protein-protein interactions of the whole genome can be evaluated, while ENRICHR is a gene enrichment analysis tool. The gene enrichment analysis sorted the hits into functional groups to see the what genes and pathways were affected by treatment. We selected the proteins for the future validation based on the known interactions and described protein functions.

The results showed that the drugs disturbed several functional pathways. For example, both volasertib and NMS-1286937 affected the key proteins in fertility and immune system regulation (KIF1BP), histones and chromatin remodeling (HP1BP3) lipid metabolism (LSS), or heme biosynthesis (PPOX). Furthermore, BI2536 and volasertib overlapped on the effect on enzymes involved in prostaglandin (PTGR2) or phosphatidyl-inositol metabolism (PIP4K2C), cell proliferation (FNTB), fertility regulation (PPT1) and RNA processing (PHF5A, NUDT1). Notably, not all changes in thermal profiles occur due to a direct effect of inhibitors. Thermal shifts can also occur as a downstream effect after Plk1 destabilization. For example, we can assume that this might be the case for MZT1 that can be affected as a downstream target of Plk1.

Febrile neutropenia and sepsis were among the most common reported adverse reactions in patients treated in the phase I-III clinical trials with Plk1 inhibitors and volasertib in particular (**Figure 7**). [148,184,185] Therefore, we decided to investigate which proteins from our hit list could potentially contribute to the disruption of the immune function.

One of the proteins that was affected by both BI2536 and volasertib was PTGR2. It is an enzyme, that is involved in prostaglandin E_2 (PGE_2) metabolism. PGE_2 is synthesized from arachidonic acid by enzymatic activity of cyclooxygenases 1 and 2 (COX1 and COX2). PGE_2 is known for its crucial role in the inflammatory response by modulating cytokine production, and was shown to contribute to sepsis development. [186,187] We hypothesized that in case of PTGR2 inhibition, the reversible mechanism of PGE_2 metabolism will lead to an increase of PGE_2 concentration and potentially will contribute to a high incidence of sepsis and bacterial infection in volasertib-treated patients.

Therefore, in order to validate the hypothesis that PTGR2 is a true off-target of volasertib, one of the future steps is to run LC-MS/MS to quantitatively compare the final amount of the proteins between DMSO-, volasertib- and NMS-1286937-treated cells at clinically relevant concentrations (25 and 500 nM). The “absorption, distribution, metabolism and excretion” (ADME) study on volasertib revealed that the maximal measured concentration of the drug in plasma was 926 nM with 17.8% coefficient of variation. [188] We experimentally identified 25 nM of volasertib as a minimal toxic dose for Jurkat at 48h timepoint. Therefore, performing the validation experiments on PTGR2 with volasertib at 25 nM and 500 nM would render clinically relevant results.

Important consideration is that Jurkat is one of the most widely used cell lines for *in vitro* studies in ALL. However, as any other result from the cell lines, our data has to be carefully extrapolated to a clinical setting. Additional experiments on healthy PBMCs should be done to see the effect of the drugs on the immune response.

3.2 Papers II and III. Targeting primary cells from pediatric T-cell and B-cell ALL patients with RNAi- prodrugs

The field of modified siRNAs is quickly expanding due to their potential to target "undruggable diseases" by modulating the mRNA expression, such as rare syndromes or "single gene disorders". Despite that cancer is a complex multifactorial disease, siRNA-based therapeutics can be effectively used in targeting a gene that is important for cancer cell proliferation or survival. As Plk1 is known as an attractive anti-cancer target, crucial for cancer cell division, we decided to use RNAi against *Plk1* mRNA as a selective therapy approach.

3.2.1.1 Ethical considerations of primary cells use

Research that involves material from patients, especially children, always requires additional care. In papers II and III we used PB samples and BM aspirates, collected from pediatric ALL patients as a part of diagnostic process before the treatment. Therefore, the collection of patient samples for our projects did not require additional intervention or discomfort for the sick children.

One of the recommendations from reviewers during the publication process was to include more patients in the study. As shown in the **Figure 2A/2B** and according to the NOPHO statistics, every year approximately 200 children get ALL. [26] Therefore, there are limitations on availability of new samples from pediatric patients.

As healthy controls, we used peripheral mononuclear blood cells (PBMC) from adult healthy donors. Some reviewers suggested to avoid comparing Plk family mRNA expression between cells from patients with pediatric ALL and healthy adults due to a potential age-dependent difference in mRNA expression. However, the enrollment of healthy children in the studies and exposing them to a painful procedure has to be ethically clarified and approved. Moreover, different studies also used PBMCs from adult healthy volunteers to study pediatric leukemia. [20,136,189,190] We checked the publicly available databases for the Plk family expression to compare with our results, and found correlation in regard to Plk1, Plk2 and Plk4, however, not Plk3 expression. In conclusion, we suggested our data to be reliable for publication.

3.2.1.2 Experimental flow

First, we evaluated the mRNA expression of the Plk family in cell lines, patient samples and healthy PBMCs **(Figure 13)**. Our data supported the previous reports about a high expression of *Plk1* in cancer cells, including cell lines and primary patient cells. We also observed a significantly higher expression of *Plk4* in tumor cells compared to healthy cells, that aligned with the data about the potential role of Plk4 as an oncogene.

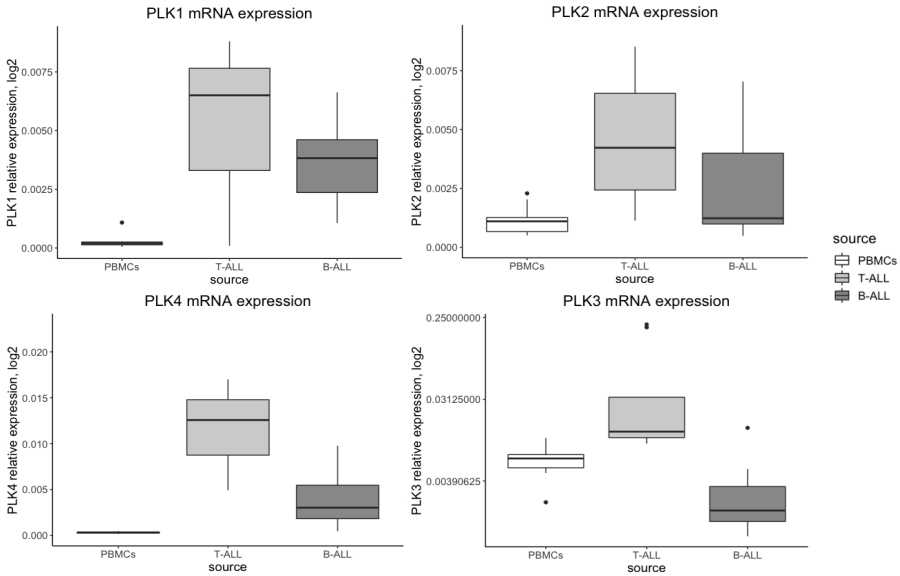

Figure 13. *Polo like kinases family expression (Plk1-Plk4) summary from the papers II and III*

In the paper III we also performed a comparative analysis of *Plk1-Plk4* mRNA expression in sorted healthy CD19+ B-cells and B-cell ALL primary patient cells derived from the PB. The data was obtained from the publicly available database R2: Genomics analysis and visualization platform. The analysis showed that the expression pattern of *Plk1* and *Plk4* followed the same trend as found in our observations of high *Plk1* and *Plk4* mRNA expression in primary cells. However, the expression of *Plk3* in the dataset was higher in B-cell ALL samples, in contrast to our data. One explanation is that the expression pattern of *Plk3* varied due to its involvement in the stress response. As seen on the **Figure 6**, the level of *Plk1* mRNA expression from the publicly available databases was shown to be higher in a BM compared to PB. BM in a healthy individual is a special environment filled with different types of rapidly dividing cells, therefore a high expression of *Plk1* was observed.

The identification of a high expression of *Plk1* in the patient cells indicated that Plk1 was a promising target to knockdown with siRNNs. However, handling of the primary cells usually requires additional stimulation with growth factors or interleukins **(Figure 14)**. In the paper II we combined anti-biotin MACSiBead Particles with biotinylated human CD2/CD3/CD28 antibodies with an addition of IL2 **(Figure 14)**. Loading of the particles with the antibodies is required to mimic the antigen-presenting cells (APCs) and to activate resting T-cells. Anti-CD3 binds to CD3 receptor and activates CD3-TCR complex. The manufacturer recommends using MACSiBead Particles to activate healthy T-cells, however, we successfully applied similar strategy for primary cancer cells. In the paper III we activated the B-cells with CD40Ligand (CD40L) and a mix of cytokines IL2, IL4 and IL7. CD40 receptor is expressed on B-cells in ALL, and CD40L mimics a T-cell dependent activation of naive B-cells and helps to stimulate the B-cell to enter the cell cycle. [191,192] IL2 and IL7 increase B-cell proliferation and IL7 alone is important for *in vivo* B-cell maturation. [193] After or simultaneously with the activation, we treated the primary cells with siRNNs.

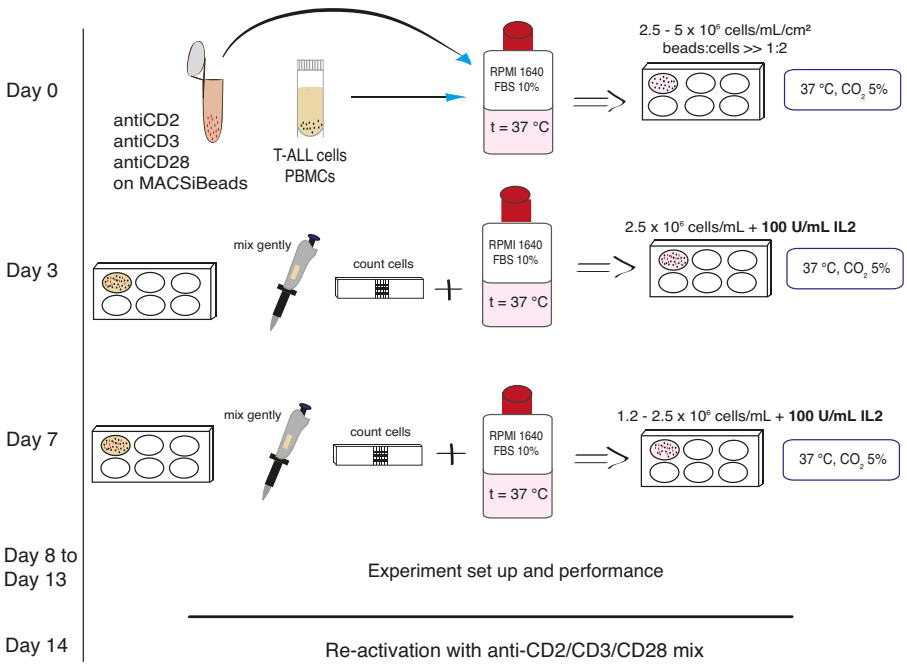

Figure 14. *Primary T-cell activation protocol of the cells, applied in the paper II.*

RNAi-based therapies are relatively novel, therefore, the experiments on siRNA-based drugs have to fulfill certain criteria in order to gain technically sound results. [162] For example, one of the initial steps in the Plk1 siRNN development (prior to the start of the studies for the papers II and III) included evaluation of six different *Plk1*-targeting siRNA sequences. [167] Out of the six, two sequences induced the highest percentage of G2 arrest in U2OS cells, what was repeated in our lab with siRNA for the paper III. Finally, one sequence was chosen for future work.

A transfection reagent (i.e. lipofectamin) is necessary for siRNA to be transfected inside the cell due to its large size and negative charge. In contrast, the modifications applied to siRNN such as TAT-peptide, phosphotriester links and 2'- modifications (2'-F and 2'-*O*-Me) allow the intracellular self-delivery via endocytosis. In our experiments, we used si-negative sequence as a control for siRNA and short interfering Luciferase (Luc siRNN) sequence as a control for Plk1 siRNN. One of the issues that is still has to be solved is a relative toxicity of Luc siRNN towards patient cells.

To evaluate and validate the efficiency of transfection with siRNA and treatment with siRNN, we used methods such as real time – quantitative polymerase chain reaction (RT-qPCR), WB, cell cycle analysis, apoptosis evaluation by staining with Annexin V (AxV)/propidium iodide (PI) staining, and cell viability assay. Treatment of the cell lines and successful activation of the primary T-ALL cells (paper II) allowed to perform at least two or three independent experiments with siRNNs and obtain the results from all the above-mentioned methods. However, this was not possible in the paper III due to a low number of primary cells in each patient sample. Overall, we analyzed the siRNNs in the cell lines and primary patient cells and succeeded to obtain statistically significant *Plk1* knockdown.

Simultaneously, we assessed the specificity of siRNNs by measuring a relative mRNA expression of *Plk2*, *Plk3* and *Plk4*. As a functional assay, we used RT-qPCR to show an increase in Mediator of DNA damage checkpoint 1 (MDC1), a marker of dsDNA breaks (paper III). The group of histones H2 is involved in a post-translational modification of chromatin and H2 phosphorylation is a DNA damage marker. MDC1 is recruited by ataxia telangiectasia-mutated (ATM) kinase to phosphorylated gamma H2AX (pH2AX), and in turn, MDC1 recruits ATM kinase together with multiple proteins to the break point and form a so-called foci, what is required for the further checkpoint activation, damage repair or histone ubiquitination. [194,195]

In the experiments in the papers II and III we had to ensure the decrease in Plk1 protein level and show that *Plk1* mRNA knockdown induced a programmed cell death – apoptosis in the cancer cells. In our studies, we used WB to detect apoptosis markers such as cleaved Poly (ADP ribose) polymerase (PARP) and G2/M

arrest marker phospho-histone H3 (pH3). PARP cleavage occurs in case of a vast DNA damage and apoptosis, that is catalyzed by caspase 3. [196] Phosphorylation of the histone H3 (pH3) on the Ser10 residue is another G2/M arrest and apoptosis marker. [197] Therefore, the expression of these proteins supported the data that *Plk1* mRNA cleavage results in G2/M arrest and apoptosis. It has also been shown that *Plk1*-depleted cells after dsDNA damage are unable to continue mitotic progression, therefore we evaluated the levels of pH2AX using WB to confirm the results from RT-qPCR on DNA breaks induction upon treatment with Plk1 siRNN.

Cell cycle analysis with PI in the primary cells of T-cell ALL patients and PBMCs from the healthy donors (paper II) was used to show the redistribution of the cell cycle phases (G1, S and G2/M) after stimulation. In the paper III, we confirmed the cell cycle arrest in G2/M after treatment with two different Plk1 siRNA sequences in U2OS cell line and G2/M arrest after Plk1 siRNN treatment in 697 and SupB15 cell lines. Due to technical limitations, we could not run the cell cycle analysis on the patient cells, however, high *Plk1* mRNA expression in unstimulated samples gave an indication that cells were viable and entered the cell cycle after thawing.

AxV/PI staining is used to measure the number of apoptotic and necrotic cells using flow cytometry. AxV staining detects the presence of phosphatidylserine on the cell surface and indicates the disrupted permeability of the cell membrane. In case of severe damage, PI can cross the cell membrane and stain the nucleus, indicating late apoptosis and necrosis of the cells. In paper II we showed that Plk1 siRNN increases the number of AxV-positive population in primary cells, while in paper III we were able to use this method only on cancer cell lines.

Overall, our data provides basis for the future *in vivo* experiments with Plk1 siRNN. Meade *et al*. showed that TAT-siRNN did not induce IFNα production in PBMCs, while *in vivo* delivery of GalNAc-siRNN induced a strong RNAi response. [167] The question of side effects in case of *in vivo* Plk1 mRNA knockdown is important to consider due to Plk1 functions and the potential as an anticancer target, therefore Raab *et al*. created an *in vivo* model with doxycycline – induced shRNA *Plk1* knockdown. [198] It was shown that *Plk1* silencing did not lead to a BM suppression, keeping normal levels of WBCs and erythrocytes compared to the wild type mice. The only difference was a decreased ferritin level. Moreover, silenced *Plk1* did not affect the growth of primary healthy cells such as fibroblasts, keratinocytes and endothelial cells. These data indicated that *Plk1* silencing leads to the tumor-selective apoptosis and does not affect normal cells.

Therefore, if you ask, “Is there a clinical future in Plk1 siRNNs?”, I will say “Yes”.

4 FUTURE PERSPECTIVES

In 2015 the European Society of Pediatric Oncology (SIOPE), together with several international collaborators, developed and published a strategy to "cure more and cure better childhood cancer by 2030" **(Figure 15)**. [199] From this perspective, the current thesis contributed to two strategic steps.

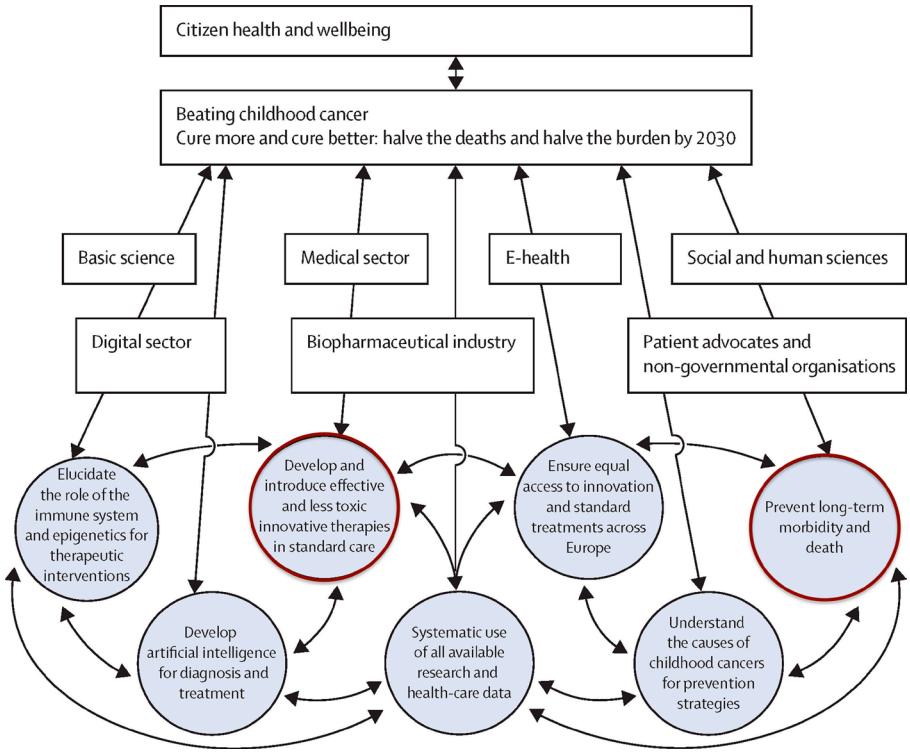

Figure 15. *EU pediatric cancer mission, proposal from European Society of Paediatric Oncology (SIOPE). Adaptation of the framework presented by Mariana Mazzucato in "Mission – oriented research & innovation in the European Union"* [200]

The first step is to develop a more effective and less toxic treatment. Despite that small molecule inhibitors have already been used in clinics, there is still a great need for other therapies in the cancer management including RNAi therapeutics. The delivery methods of siRNA-based drugs are extensively improving, allowing specific and non-immunogenic treatment of blood malignancies. Another therapeutic challenge is related to resistant patients. However, it is still unclear if the level of Plk1 expression influences tumor resistance and if the primary/secondary

resistance mechanism will affect the intracellular delivery of siRNA-based drugs. Therefore, more studies are needed to evaluate the potential of using RNAi-based therapies in resistant/relapsed patients.

The second step aims to reduce long-term consequences from toxicities during and after the treatment. Paper I showed that one drug alone can change the whole landscape of the cellular processes in the body, not always for the benefit of the patient. Thus, more studies on the anticancer drug off-targets will help to predict potential complications and likely help to switch to the more efficient protocols.

As for now, Plk1 is a promising target for patients with different types of cancers and leukemia in particular. Analysis and comparison of the adverse reactions that occur after treatment with small molecule inhibitors and novel RNAi-based therapies, such as givosiran, suggest that siRNA-based drugs can potentially exhibit lower toxicity. However, in case of Plk1 siRNN, *in vivo* studies and computational modelling are needed to predict the potential effect of treatment and possible combinations to ensure synthetic lethality in cancer cells.

It is interesting to observe how scientific developments change the treatment approaches and expand the field of personalized medicine. Doctors have to keep in mind that every day we are getting closer to the opportunity to treat each unique patient individually. The clonal variability of tumors requires treatment with several drugs with different mode of action. The development of novel combination protocols i.e. with monoclonal antibodies, with revised doses of chemotherapeutical drugs and with targeted oligonucleotides-based therapies is one of the tasks of personalized medicine. This approach can potentially decrease the duration of treatment, reduce severity of the adverse reactions and improve outcomes in patients. The increased quantity and quality of data from genome-wide association studies and development of artificial intelligence might also contribute to a better treatment of cancer patients.

Another crucial point in the future considerations is that RNAi therapies require not only optimizing the intracellular delivery methods, but also set a challenge of manufacturing processes of the large amounts of modified siRNAs, quality controls and delivery to a patient. This field requires a collaborative approach in terms of discovery, evaluation, and development of the infrastructure to produce the drug and treat a patient.

In conclusion, the present thesis contributes to the fight against a pediatric cancer specifically by adding a brick of information to prevent long-term consequences of treatment in patients with acute leukemia and introducing innovative effective and less toxic treatments.

5 POPULAR SCIENCE SUMMARY

Leukemia is a form of blood cancer that very often affects children. Currently, to treat one type of leukemia – acute lymphoblastic leukemia – doctors use a combination of different chemotherapeutic drugs that are toxic to cancer cells and the normal cells of the body. This toxicity leads to severe side effects during the treatment and many years after the completion of the therapy. This issue is especially important for children that survive cancer, because in the long-term perspective they have an increased risk of secondary tumors, infertility, mental and/or heart problems. Therefore, there is a permanent search for new treatments that are both efficient against cancer and have less side effects.

During the recent years it became clear that RNA has many more functions than just being a transcript for a protein synthesis. One of the approaches that our bodies developed evolutionary to silence the alien RNA, for example from viruses, is called RNA interference (RNAi). In the light of developing gene therapies, it became evident that specific messenger RNAs (mRNA) can be used as targets to silence the translation of the proteins important for the cancer cell proliferation and growth. RNAi happens inside every cell, normal and cancer, and is induced by both endogenous and alien RNA. Once inside the cell, special enzymes can cut the alien RNA into short interfering (si)RNA, that connects to specific proteins to form a so-called RNA-induced silencing complex, or RISC. This complex induces a cascade of events that result in elimination of a protein-coding mRNA. Lack or absence of the protein leads to the lack of function. Scientists use this method not only for learning and understanding the function of different genes and proteins, but also to treat diseases, including cancer.

Plk1 is an important protein that helps any cell to divide. Without Plk1 cancer cells cannot grow and die via a specific cell death process called apoptosis. Thus, Plk1 was identified as a good target for anticancer treatment, and pharmaceutical companies have developed several small molecule drugs that block the function of Plk1. Unfortunately, the tested drugs have induced many side effects in patients, and in one of our studies we investigated the potential off-target proteins of these drugs and which of the proteins could be responsible for the unfavorable outcomes in clinical trials.

We also analyzed a modified siRNA molecule named siRNN that targets Plk1 and showed that leukemic cells that were taken from the patients and put in culture die after our treatment. Moreover, our modified drug did not cause any changes in synthesis of other proteins that belong to the same family as Plk1 and proved to be less toxic towards normal blood cells compared to small molecule drugs.

Therefore, Plk1 siRNN can be further investigated and can be implemented in the future for acute leukemia treatment.

In a nutshell, this thesis has improved the understanding of side effects caused by the small molecule inhibitors against Plk1 and introduced a novel drug that is more selective and might cause less side effects in children with ALL. Future studies will help to understand better what group of patients will benefit the most from the proposed treatment and what combinations of drugs should be used.

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In the beginning was the Word...
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The last and the most important words I am saying to my great boyfriend. I am grateful for all your life choices and acceptance of who I am. Thank you for your love, mindfulness, and support, no matter how different our sense of humor is ;) Together we will definitely conquer this world!

I love you.

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